NTP TECHNICAL REPORT

ON THE

# TOXICOLOGY AND CARCINOGENESIS

# STUDIES OF *p*-NITROTOLUENE

(CAS NO. 99-99-0)

# IN F344/N RATS AND B6C3F$_1$ MICE

(FEED STUDIES)

NATIONAL TOXICOLOGY PROGRAM
P.O. Box 12233
Research Triangle Park, NC 27709

May 2002

NTP TR 498

NIH Publication No. 02-4432

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

## FOREWORD

The National Toxicology Program (NTP) is made up of four charter agencies of the U.S. Department of Health and Human Services (DHHS): the National Cancer Institute (NCI), National Institutes of Health; the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health; the National Center for Toxicological Research (NCTR), Food and Drug Administration; and the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control and Prevention. In July 1981, the Carcinogenesis Bioassay Testing Program, NCI, was transferred to the NIEHS. The NTP coordinates the relevant programs, staff, and resources from these Public Health Service agencies relating to basic and applied research and to biological assay development and validation.

The NTP develops, evaluates, and disseminates scientific information about potentially toxic and hazardous chemicals. This knowledge is used for protecting the health of the American people and for the primary prevention of disease.

The studies described in this Technical Report were performed under the direction of the NIEHS and were conducted in compliance with NTP laboratory health and safety requirements and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals. The prechronic and chronic studies were conducted in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations, and all aspects of the chronic studies were subjected to retrospective quality assurance audits before being presented for public review.

These studies are designed and conducted to characterize and evaluate the toxicologic potential, including carcinogenic activity, of selected chemicals in laboratory animals (usually two species, rats and mice). Chemicals selected for NTP toxicology and carcinogenesis studies are chosen primarily on the bases of human exposure, level of production, and chemical structure. The interpretive conclusions presented in this Technical Report are based only on the results of these NTP studies. Extrapolation of these results to other species and quantitative risk analyses for humans require wider analyses beyond the purview of these studies. Selection *per se* is not an indicator of a chemical's carcinogenic potential.

Details about ongoing and completed NTP studies are available at the NTP's World Wide Web site: http://ntp-server.niehs.nih.gov. Abstracts of all NTP Technical Reports and full versions of the most recent reports and other publications are available from the NIEHS' Environmental Health Information Service (EHIS) http://ehis.niehs.nih.gov (800-315-3010 or 919-541-3841). In addition, printed copies of these reports are available from EHIS as supplies last. A listing of all the NTP Reports printed since 1982 appears on the inside back cover.

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# CONTENTS

## Summary

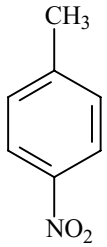
**Background:** Approximately 15 million pounds of *para*-nitrotoluene are used annually in the United States in the production of agricultural and rubber chemicals and dyes. We studied the effects of *p*-nitrotoluene on male and female rats and mice to identify potential toxic or carcinogenic hazards to humans.

**Methods:** We gave feed containing 1,250, 2,500, or 5,000 parts per million (ppm) *p*-nitrotoluene (equivalent to 0.125%, 0.25%, or 0.5%) to groups of 60 male and female rats and mice for 2 years. Groups of animals receiving untreated feed served as controls. Tissues from more than 40 sites were examined for every animal.

**Results:** All of the groups fed 5,000 ppm *p*-nitrotoluene weighed less than the controls. Significantly more clitoral gland tumors occurred in female rats receiving 2,500 ppm than in the control group. There were more subcutaneous fibromas and fibrosarcomas in male rats fed *p*-nitrotoluene and more lung tumors in male mice fed *p*-nitrotoluene than in the controls.

**Conclusions:** We conclude that the increased incidence of clitoral gland neoplasms in female rats was caused by exposure to *p*-nitrotoluene. Subcutaneous tumors in male rats and lung tumors in male mice may have been related to exposure to *p*-nitrotoluene.

# ABSTRACT



**_p_-NITROTOLUENE**

CAS No. 99-99-0

Chemical Formula: $C_7H_7NO_2$ Molecular Weight: 137.14

**Synonyms:** Methyl nitrobenzene; 1-methyl-4-nitrobenzene; 4-methylnitrobenzene; *p*-methylnitrobenzene; *p*-nitrophenylmethane; 4-nitrotoluol; 4-nitrotoluene; PNT

*p*-Nitrotoluene is used to synthesize agricultural and rubber chemicals, azo and sulfur dyes, and dyes for cotton, wool, silk, leather, and paper. *p*-Nitrotoluene was nominated by the National Institute for Occupational Safety and Health and the NTP for study based on its considerable human exposure as well as the absence of long-term studies of its carcinogenicity in rodents. Male and female F344/N rats and B6C3F$_1$ mice were exposed to *p*-nitrotoluene (greater than 99% pure) in feed for 2 years. Genetic toxicology studies were conducted in *Salmonella typhimurium*, L5178Y mouse lymphoma cells, cultured Chinese hamster ovary cells, and rat and mouse bone marrow cells.

## 2-Year Study in Rats

Groups of 50 male and 50 female rats were fed diets containing 0, 1,250, 2,500, or 5,000 ppm *p*-nitrotoluene (equivalent to average daily doses of approximately 55, 110, or 240 mg *p*-nitrotoluene/kg body weight to males and 60, 125, or 265 mg/kg to females) for 105 or 106 weeks.

### *Survival, Body Weights, and Feed Consumption*

Survival of all exposed groups of rats was similar to that of the control groups. Mean body weights of 5,000 ppm male and 2,500 and 5,000 ppm female rats were less than those of the controls during most of the study; mean body weights of 1,250 ppm females were less during the second year of the study. Feed consumption by 5,000 ppm females was less than that by the controls during year 2 of the study.

### *Biomarkers of Exposure*

Two urinary metabolites were followed during the study as biomarkers of exposure. The ratios of *p*-nitrobenzoic acid to creatinine and of *p*-acetamidobenzoic acid to creatinine determined at 2 weeks and at 3, 12, and 18 months were linearly related to exposure concentration in males and females.

### *Pathology Findings*

The incidence of clitoral gland adenoma or carcinoma (combined) was significantly greater in 2,500 ppm

females than that in the controls and exceeded the historical control ranges. The incidence of clitoral gland neoplasms was not increased in 5,000 ppm females, possibly because of the lower body weights in this group. The incidences of subcutaneous fibroma and of subcutaneous fibroma or fibrosarcoma (combined) in 2,500 ppm male rats were significantly increased and exceeded the historical control ranges.

The incidences of several nonneoplastic kidney lesions were significantly increased in exposed groups of rats, and the severities of these lesions generally increased with increasing exposure concentration. In the spleen, incidences of hematopoietic cell proliferation and pigmentation were significantly increased in the 2,500 and 5,000 ppm groups. Significantly increased incidences of various types of altered cell foci in the liver of males and females were associated with exposure. Incidences of germinal epithelial atrophy of the testis in 5,000 ppm males and endometrial cystic hyperplasia of the uterus in 2,500 and 5,000 ppm females were significantly increased.

The incidences of mononuclear cell leukemia were significantly decreased in all exposed groups except 1,250 ppm females. The incidence of interstitial cell adenoma of the testis in 5,000 ppm males was significantly decreased.

## 2-Year Study in Mice

Groups of 50 male and 50 female mice were fed diets containing 0, 1,250, 2,500, or 5,000 ppm *p*-nitrotoluene (equivalent to average daily doses of approximately 170, 345, or 690 mg/kg to males and 155, 315, or 660 mg/kg to females) for 105 or 106 weeks.

### *Survival, Body Weights, and Feed Consumption*

Survival of all exposed groups of male and female mice was similar to that of the control groups. Mean body weights of 5,000 ppm males and females were less than those of the control groups during most of the study. Mean body weights of 2,500 ppm males were less than those of the controls after week 92. Feed consumption by all exposed groups of mice was similar to that by the control groups.

### *Pathology Findings*

The incidence of alveolar/bronchiolar adenoma or carcinoma (combined) was significantly greater in 5,000 ppm male mice than in the controls, as was the incidence of alveolar epithelial hyperplasia in this group. The incidences of alveolar epithelial bronchiolization were significantly increased in all exposed groups of males and females.

## Genetic Toxicology

*p*-Nitrotoluene was not mutagenic in any of several strains of *S. typhimurium*, with or without metabolic activation enzymes (S9). A positive response to *p*-nitrotoluene was observed in the L5178Y mouse lymphoma cell assay in trials with S9. Significantly increased sister chromatid exchange frequencies were observed in cultured Chinese hamster ovary cells treated with *p*-nitrotoluene with and without S9. Chromosomal aberrations were also induced in Chinese hamster ovary cells treated with *p*-nitrotoluene in the presence of S9; no increased aberrations were seen without S9. *p*-Nitrotoluene did not induce a significant reproducible increase in the frequency of micronuclei in bone marrow polychromatic erythrocytes of male rats or male mice when administered by intraperitoneal injection.

## Conclusions

Under the conditions of these 2-year feed studies there was *equivocal evidence of carcinogenic activity** of *p*-nitrotoluene in male F344/N rats based on increased incidences of subcutaneous skin neoplasms. There was *some evidence of carcinogenic activity* of *p*-nitrotoluene in female F344/N rats based on increased incidences of clitoral gland neoplasms. There was *equivocal evidence*

*of carcinogenic activity* of *p*-nitrotoluene in male $B6C3F_1$ mice based on increased incidences of alveolar/bronchiolar neoplasms. There was *no evidence of carcinogenic activity* of *p*-nitrotoluene in female $B6C3F_1$ mice exposed to 1,250, 2,500, or 5,000 ppm.

Exposure to *p*-nitrotoluene caused increased incidences of nonneoplastic lesions of the kidney, spleen, and liver in male and female rats, testis in male rats, and lung in male and female mice.

Decreased incidences of mononuclear cell leukemia in male and female rats and testicular interstitial cell adenoma in male rats were attributed to exposure to *p*-nitrotoluene.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 10. A summary of the Technical Reports Review Subcommittee comments and public discussion on this Technical Report appears on page 12.

**Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of *p*-Nitrotoluene**

| | Male F344/N Rats | Female F344/N Rats | Male $B6C3F_1$ Mice | Female $B6C3F_1$ Mice |
|---|---|---|---|---|
| **Concentrations in feed** | 0, 1,250, 2,500, or 5,000 ppm | 0, 1,250, 2,500, or 5,000 ppm | 0, 1,250, 2,500, or 5,000 ppm | 0, 1,250, 2,500, or 5,000 ppm |
| **Body weights** | 5,000 ppm group less than the control group | Exposed groups less than the control group | 2,500 and 5,000 ppm groups less than the control group | 5,000 ppm group less than the control group |
| **Survival rates** | 31/50, 38/50, 38/50, 40/50 | 39/50, 37/50, 39/50, 41/50 | 46/50, 46/50, 45/50, 42/50 | 46/50, 47/50, 43/50, 49/50 |
| **Nonneoplastic effects** | Kidney: renal tubule hyaline droplet (2/50, 23/50, 27/50, 18/50); renal tubule pigmentation (10/50, 28/50, 47/50, 46/50)<br>Spleen: hematopoietic cell proliferation (9/50, 13/50, 19/50, 25/50); pigmentation (10/50, 12/50, 24/50, 38/50)<br>Liver: basophilic focus (31/50, 39/50, 42/50, 45/50); clear cell focus (20/50, 27/50, 30/50, 32/50); eosinophilic focus (5/50, 5/50, 5/50, 19/50)<br>Testis: germinal epithelial atrophy (7/50, 11/50, 8/50, 30/50) | Kidney: renal tubule hyaline droplet (8/50, 41/50, 49/50, 46/50); renal tubule pigmentation (9/50, 43/50, 49/50, 50/50); mineralization (15/50, 21/50, 32/50, 40/50); oncocytic renal tubule hyperplasia (0/50, 2/50, 4/50, 6/50)<br>Spleen: hematopoietic cell proliferation (26/50, 26/50, 45/50, 43/50); pigmentation (24/50, 32/50, 45/50, 48/50)<br>Liver: eosinophilic focus (1/50, 2/50, 7/50, 9/50) | Lung: alveolar epithelial bronchiolization (0/50, 20/50, 30/50, 48/50); alveolar epithelial hyperplasia (1/50, 1/50, 4/50, 6/50) | Lung: alveolar epithelial bronchiolization (0/50, 33/50, 41/50, 49/50) |
| **Neoplastic effects** | None | Clitoral gland: adenoma or carcinoma (8/50, 12/50, 20/50, 8/49) | None | None |
| **Equivocal findings** | Skin (subcutaneous): fibroma (1/50, 2/50, 7/50, 1/50); fibroma or fibrosarcoma (1/50, 2/50, 9/50, 1/50) | None | Lung: alveolar/ bronchiolar adenoma or carcinoma (8/50, 14/50, 12/50, 19/50) | None |

**Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of *p*-Nitrotoluene**

| | Male F344/N Rats | Female F344/N Rats | Male $B6C3F_1$ Mice | Female $B6C3F_1$ Mice |
|---|---|---|---|---|
| **Decreased incidences** | Mononuclear cell leukemia: (24/50, 12/50, 5/50, 4/50)<br>Testis: interstitial cell adenoma (49/50, 46/50, 45/50, 34/50) | Mononuclear cell leukemia: (13/50, 12/50 3/50, 1/50) | None | None |
| **Level of evidence of carcinogenic activity** | Equivocal evidence | Some evidence | Equivocal evidence | No evidence |
| **Genetic toxicology** | | | | |
| *Salmonella typhimurium* gene mutations: | Negative in strains TA98, TA100, TA1535, and TA1537 with and without S9 | | | |
| Mouse lymphoma gene mutations: | Positive with S9, negative without S9 | | | |
| Sister chromatid exchanges | | | | |
| Cultured Chinese hamster ovary cells *in vitro*: | Positive with and without S9 | | | |
| Chromosomal aberrations | | | | |
| Cultured Chinese hamster ovary cells *in vitro*: | Positive with S9, negative without S9 | | | |
| Micronucleated erythrocytes | | | | |
| Rat bone marrow *in vivo*: | Negative when administered by intraperitoneal injection | | | |
| Mouse bone marrow *in vivo*: | Negative when administered by intraperitoneal injection | | | |

## EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results **(clear evidence and some evidence);** one category for uncertain findings **(equivocal evidence);** one category for no observable effects **(no evidence);** and one category for experiments that cannot be evaluated because of major flaws **(inadequate study).** These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

For studies showing multiple chemical-related neoplastic effects that if considered individually would be assigned to different levels of evidence categories, the following convention has been adopted to convey completely the study results. In a study with clear evidence of carcinogenic activity at some tissue sites, other responses that alone might be deemed some evidence are indicated as "were also related" to chemical exposure. In studies with clear or some evidence of carcinogenic activity, other responses that alone might be termed equivocal evidence are indicated as "may have been" related to chemical exposure.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or in other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

## NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS
## TECHNICAL REPORTS REVIEW SUBCOMMITTEE

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Technical Report on *p*-nitrotoluene on May 3, 2001 are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing the NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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---

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## SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On May 3, 2001, the draft of the Technical Report on the toxicology and carcinogenesis studies of *p*-nitrotoluene received public review by the National Toxicology Program's Board of Scientific Counselors' Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. J.K. Dunnick, NIEHS, introduced the toxicology and carcinogenesis studies of *p*-nitrotoluene by discussing the uses of the chemical and the rationale for the study, describing the experimental design, reporting on survival and body weight effects, and commenting on chemical-related neoplasms in male and female rats and mice. The proposed conclusions were *equivocal evidence of carcinogenic activity* in male F344/N rats, *some evidence of carcinogenic activity* in female F344/N rats, *equivocal evidence of carcinogenic activity* in male $B6C3F_1$ mice, and *no evidence of carcinogenic activity* in female $B6C3F_1$ mice.

Dr. Davis, the first principal reviewer, questioned the use of the term uncertain findings to describe conclusions of equivocal evidence. He disagreed with the statement in the report that hematopoietic cell proliferation increased in the 5,000 ppm rats. Dr. Dunnick concurred. Dr. Davis also questioned whether there could be a relation between testicular interstitial cell adenomas and atrophy when the incidences of the former decreased while the latter increased. Dr. J. Mahler, NIEHS, explained that while atrophy can occur as a secondary effect of an adenoma, the absence of a neoplasm may increase the possibility of detecting a primary atrophic change. Dr. Davis also encouraged the inclusion of human exposure data whenever available.

Dr. Medinsky, the second principal reviewer, was unable to attend the meeting, and her comments were read into the record by Dr. M.S. Wolfe, NIEHS. Dr. Medinsky agreed with the proposed conclusions and focused on details of the discussion of metabolism and urinary biomarker data. Dr. Dunnick indicated that communications between NTP staff and Dr. Medinsky had resolved these questions.

Dr. Klaunig, the third principal reviewer, asked about the cause of apparent lower survival in control male rats compared with the high dose males. Dr. Dunnick noted that the survival in control male rats was normal for NTP studies, with mononuclear cell leukemia being one of the main causes of early deaths. However, in the exposed animals, splenic toxicity caused by the chemical inhibited the occurrence of mononuclear cell leukemia.

Dr. Davis moved that the conclusions be accepted as written and Dr. Klaunig seconded the motion, which was approved unanimously with eight votes.